Protein Permeability Through Thin Organic Layers[1]

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IT HAS BEEN KNOWN for several years (*1, 2, 14*) that specific adsorption of antibody molecules up to several hundred angstrom units thick takes place when a plate covered with molecular layers of antigenic protein is treated for a few minutes with diluted antiserum. With normal or heterologous serum only a very small nonspecific adsorption amounting to 10–20 A occurs.

In 1942, Rothen and Landsteiner (*14*) made the following observations:

When, after transfer of an antigen film to a metal slide, a double layer of stearic acid-stearate was deposited on top of the antigenic protein, there was the normal increase of 48 A in thickness, but the plate came out wet instead of dry.[2] On treatment with a homologous antiserum unexpectedly a specific increase, 30 to 40 A, was observed; after covering with three double layers of stearic acid, instead of one as above, this effect no longer took place. Since a reaction between antigen and antibody, in spite of interposed layers, would seem improbable, one must consider that perhaps there were discontinuities or uncovered areas in the stearic acid film, or that a displacement had occurred which would bring the protein film above the stearic layer.

Since then Rothen has published a series of papers (*7–9–11*) on this interaction between protein molecules through screening films in which he abandoned the original explanation. His experimental evidence and considerations of related facts lead him to the postulate of long range forces between certain types of protein molecules. He states (*13*): "This long range interaction might take place through resonating extended oscillators, the presence of which is likely to occur in large molecules as suggested by London."

Considering the evidence for the necessity of close contact between specific groups of antigen and antibody molecules put forth by Pauling (*5, 6*) and others (*3, 4*) and considering the activation energy of many kilocalories necessary for the breakup of peptide bonds by the action of trypsin,[3] it is difficult to accept Rothen's long range forces explanation.

By following his basic experimental procedure, we have found it possible to reproduce all the main points of his published data. Certain deviations from Rothen's technique are listed below. The question to be resolved is not whether Rothen's experiments are correct but whether his proposed explanation is valid.

Assuming that there are no long range forces, but some kind of protein permeability of the screening material, we should expect better protection by multiple layers of screening material than by single layers of the same total thickness. By analogy, a set of sieves with random hole distribution placed tightly on top of each other is more impermeable than a single thick sieve with the same hole distribution. Our experimental results with multiple screens consisting of more than three layers of Formvar confirm strongly the validity of such an assumption, with the exception that a purely geometrical explanation is too simple.

With very thin single or multiple Formvar screens, the specific adsorption from antiserum is usually as high as, or even higher than, without screen. This is due to the fact that the leaching of antigenics material (see below) occurs faster than the formation of specific precipitate with the respective concentrations of buffer and antiserum used. The experimental evidence for this statement will be given elsewhere. Obviously, the presence of a very thin screen slows the leaching process without interfering too much with the specific reaction.

At another point in our investigation, we realized that no data had been presented by Rothen on the action of the saline phosphate buffer itself on a screen-covered antigen plate. Saline phosphate buffer was used for diluting the sera in all his experiments as well as ours. This control experiment was made and immediately suggested a simple explanation of the Rothen effect for the antigen-antibody system.

As shown in the experimental part, saline phosphate buffer or phosphate buffer removes antigen material from underneath the screen, apparently through holes.[4] If the plates are treated with distilled water, even for 40 minutes, there is no loss of antigenic material. Veronal buffer, as used in the trypsin experiments, removes antigenic material to a much smaller extent and only through very thin screens.

In experiments with saline phosphate buffer or phosphate buffer, antigen penetrated the screen and could be removed from the plate by washing with dis-

[1] Grateful acknowledgment is made of the generous assistance given by Dr. A. Rothen in this investigation.

[2] A metal plate covered only with several layers of stearic acid-stearate is water-shedding.

[3] The system trypsin-substrate has an activation energy of 10–15 kg-cal/mole, depending on the substrate.

[4] During a conversation on 12 September 1949, the author learned that Dr. Rothen had discovered independently the leaching effect of phosphate ions.

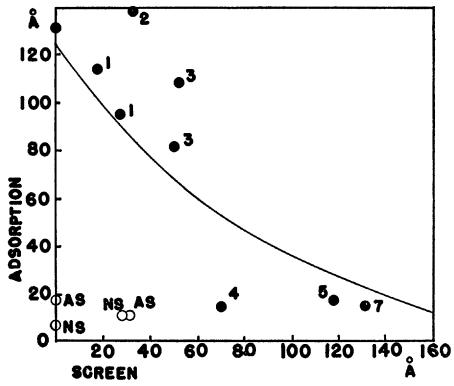

FIG. 1. Adsorption of rabbit antibody through Formvar screen on two double layers of serum albumin. Abscissas give total screen thickness. Inserted figures indicate the numbers of individual Formvar films which build up the screen. Each point represents the average of between four and eight individual experiments done under identical conditions. The curve is a reproduction of Rothen's curve for the same conditions. Our points, however, represent the true increment, i.e., we did not deduct a certain amount of non-specific adsorption, as Rothen did in his curve. Depending on the screen thickness, 5–20 A has to be deducted from our points for comparison with Rothen's curve (see Fig. 4). *AS* and *NS* represent adsorption from antiserum and normal serum on plates without antigen.

tilled water. After drying the plate, the decrease in thickness was easily measurable with an ellipsometer.[5] The amount of decrease is a function of time. Treatment of the plate thereafter with antiserum yielded a correspondingly smaller specific adsorption. When the plate is directly treated with diluted antiserum instead of saline buffer, the antigen meets the antibody as soon as it comes to the screen surface, and forms a precipitate which cannot be removed from the plate by washing in the usual way. When the plate was pretreated with diluted normal serum instead of saline buffer, the leaching effect was much stronger in case of very thin screens or on plates without screen. For thicker screens, however, this difference did not exist. Nevertheless, the treated part of the plate showed a much smaller specific adsorption of antibody after a subsequent treatment with antiserum than the control part. Treatment with normal serum gives a combined effect of decrease by loss of antigenic material and increase by unspecific adsorption of serum proteins.

It is easily possible to demonstrate the antigen which has passed the screen by placing against the screen-covered antigen plate a normal conditioned plate with a droplet of saline phosphate buffer between the two plates. After 10 minutes an increase in thickness occurs on the second plate (pickup plate) after washing and drying. A subsequent treatment with antiserum shows a specific adsorption on the pickup plate. If this experiment is performed with diluted normal serum instead of saline phosphate buffer, there is no specific adsorption. Obviously, the antigen cannot be adsorbed under those conditions on the pickup plate.

With the enzyme system, trypsin-serum albumin, the behavior is somewhat more complicated because trypsin itself appears to be able to penetrate the screen.

In conclusion, we seem to be facing the problem of a differential protein permeability of thin organic layers rather than the long range forces postulated by Rothen. This preliminary report includes a description of deviations from Rothen's original technique and the high lights of experimental results.

Optical Base. In order to get several comparable figures at the same time under identical conditions, we did not use a single boundary line between one and three or three and five layers of barium stearate on each plate, but we used ten steps, which provided us with nine independent boundary lines for measurement by matching. To increase our sensitivity, these steps were built on a uniform base of 19.5 double layers of barium stearate so that the numbers of single layers for successive steps were: 39, 41, 43 . . . up to 57. The maximum sensitivity with such a plate is between 10 and 11 degrees of analyzer rotation for a 50-A increment in thickness. A calibration curve was made for each plate. The standard deviation of our readings (determined with sets of ten readings for each

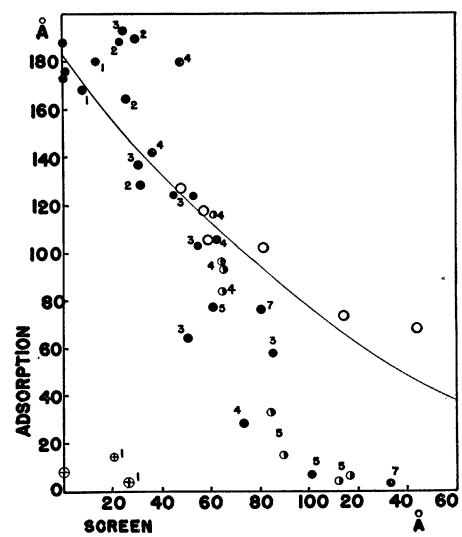

FIG. 2. Adsorption of rabbit antibody through Formvar screen on three double layers of serum albumin. Abscissas give total screen thickness. For the inserted figures and curve see the legend of Fig. 1. Open circles indicate experiments with single cast screens (Rothen's technique). Crossed circles indicate experiments on plates without antigen. Closed circles and half-closed circles represent experiments with multiple screens. The closed circles represent averages of between four and eight individual experiments, whereas the eight half-closed circles represent single readings on a plate half of whose surface was covered with four layers and the other half with five layers of Formvar.

[5] A term coined by A. Rothen for his polarization spectrometer (8).

step) was ± 0.5 A for the most sensitive part of the plate and ± 2.2 A for the least sensitive step. The experiments were made with an angle of incidence, $\varphi = 70°$. The plates were microscope slides coated with pure chromium. The optical base was always deposited on five plates simultaneously by means of a dipping device operated by oil pressure, which gave an extremely smooth and homogeneous linear movement in both directions.

Spreading of Antigen. To make sure that we did not have a loss of serum albumin into the substrate, we developed a new technique for spreading proteins which will be presented later. Usually there is an appreciable loss of protein into the substrate if a droplet of protein solution is placed on a water surface, even if this is done very carefully. This dissolved material may cause a film of undenatured protein molecules adsorbed underneath the spread protein film. For the problem under investigation it is important to avoid this error as much as possible. The thickness for three double layers of serum albumin spread by our method and deposited under a pressure of between 8 and 9 dynes per cm was always around 57 A.

Screen Casting. In case of thick single screens, we followed Rothen's original technique by dipping a clean glass slide into a solution of Formvar or collodion and depositing the dried film on a clean water surface, from which it was then transferred to the experimental plate. For thin screens we spread the solution of plastic material directly on a clean water surface and deposited it on the experimental plate by passing this plate nearly horizontally face down through the water surface, turning the plate upside down under water, cleaning the surface with a glass bar and bringing the plate up through the clean water surface. The multiple screens were all deposited in this way.

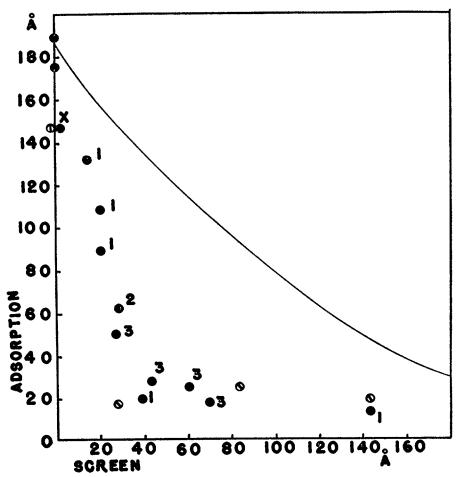

FIG. 3. Adsorption of rabbit antibody through collodion screen on three double layers of serum albumin. Abscissas give total screen thickness. Closed circles have the same meaning as in Figs. 1 and 2. Circles with oblique bar are experiments with normal serum. Circle with upright bar and the closed circle next to it (*X*) are two experiments without screen where one plate was dipped for a few seconds into pure amyl acetate and dried and then treated together with the control plate with antiserum, which in this case was a little older. This control was necessary to show that the deposition of collodion films from a substrate saturated with amyl acetate did not affect the antigenic properties of the serum albumin.

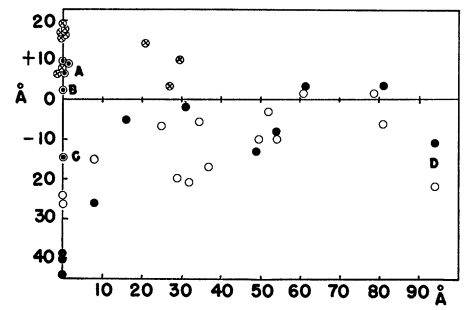

FIG. 4. Nonspecific adsorption from antiserum and action of saline phosphate buffer and normal serum. The ordinates give the increase or decrease of total thickness from plates with and without three double layers of serum albumin coated with multiple Formvar screens. The abscissas give the screen thickness. Crossed circles represent adsorption from diluted antiserum on plates without antigen. Open circles represent action of saline phosphate buffer on antigen plates. Closed circles represent action of diluted normal serum on antigen plates. Circles with black centers represent the action of diluted normal serum on plates without antigen (*A*), on plate with a single layer of antigen (*B*) and on plate with a single plus a double layer of antigen (*C*). *D* represents the action of saline phosphate buffer and diluted normal serum on antigen plate with single cast Formvar film.

Treatment with Sera. Instead of smearing a serum droplet over the slide, we deposited a serum droplet at the end of the slide and covered the plate with a microscope cover slide cut to the suitable size. The experimental area on our plates was 25 × 40 mm. The cover glasses were 22 × 50 mm. In covering the plate with this size cover glass we used 0.15 ml of diluted serum. In covering the plate with slides 9 × 40 mm, we used 0.05 ml of diluted serum. Thus the liquid film was in both cases about 135 μ thick. We believe this method of application gives more regular results. In all experiments we used a 10-minute treatment period.

Washing. We did not wash our plates after depositing the antigenic material, but we washed the plates once with saline buffer and twice with double-distilled water after treatment with sera.

Materials used. As antigen, we used crystallized bovine plasma albumin (Armour and Company) dissolved in double-distilled water. Normal serum was obtained from a healthy rabbit. The antiserum (rabbit) was kindly provided by Dr. M. Mayer, of the Johns Hopkins School of Hygiene. It contained 0.96 mg antibody-nitrogen per ml. Formvar 15/95 "E" (from Shawinigan Products Corporation in New York City) was dissolved in redistilled dichloroethane,

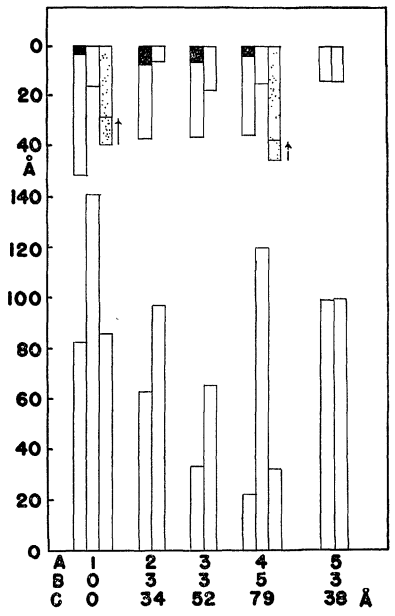

FIG. 5. Experiments with pickup plates. Ordinates represent adsorption. The diagram represents five experiments where a plate with three double layers of antigen (and multiple Formvar screen in experiments 2–5) has been covered by a conditioned plate with a droplet of saline phosphate buffer, veronal buffer, or diluted normal serum in between. A control strip was always left free. After washing and drying these plates, all plates were treated with diluted antiserum. The lower part of the diagram represents the adsorption of antibody on the five base plates after the pretreatment mentioned. The long column on each plate is always the adsorption on the control strip. The left column is the adsorption on the part which has been treated previously with saline buffer (experiments 1–4) or veronal buffer (experiment 5). In experiments 1 and 4 the right-hand column is the adsorption on the part which has been treated previously with diluted normal serum. *A* represents number of experiment, *B* represents number of single Formvar layers which build up the total screen, and *C* represents the total screen thickness in angstrom units.

The upper part of the diagram represents the findings on the pickup plates. The black sections show the actual pickup of antigen after the plates were in contact with the base plates. The dotted columns in experiments 1 and 4 show a nonspecific protein adsorption from normal serum probably mixed with antigen. The white sections and columns show the adsorption after treatment with diluted antiserum. On the two parts in experiments 1 and 4 which were treated previously with diluted normal serum, a loss of 5–10 A occurred after the antiserum treatment which is indicated by the two arrows.

Control experiments showed that a conditioned optical base does not adsorb more than the usual small amount of nonspecific material from antiserum after a 10-minute treatment with phosphate saline buffer.

and collodion U. S. P. (Merck) containing alcohol and ether was diluted by adding amyl acetate. Collodion films were spread on double-distilled water saturated with amyl acetate. Formvar films were spread on pure double-distilled water.

The first three figures represent part of our indirect evidence for the existence of a protein permeability through Formvar and collodion films. Other indirect evidence has already been presented by S. J. Singer (15). Figs. 4 and 5 represent the first direct evidence to this effect. The legends give all the necessary information.

TABLE 1

INFLUENCE OF TIME ON THE INTERACTION BETWEEN SALINE BUFFER AND A SCREEN-COVERED ANTIGEN PLATE

Time of pretreatment min	Increment after pretreatment A	Adsorption from antiserum A
0	0	142
1	−7	111
4	−20	83
8	−22	73
16	−24	70

The data in Table 1 were obtained with a plate having three double layers of serum albumin and a Formvar film of 37 A total thickness, consisting of four single Formvar layers, dipped with its longer edge horizontal to a depth four-fifths of its width into saline phosphate buffer and withdrawn by a fifth of its width at definite time intervals, so that the treatment time was 1, 4, 8 and 16 minutes. After washing with double-distilled water and drying, the plate was measured and then treated for 10 minutes with diluted antiserum as usual.

The work presented here in this paper was performed during the period April to August 1949.

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